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Urinary Survivin and the Detection of Recurrent Transitional Cell Carcinoma of the Bladder

Justin J. Cohen

YALE UNIVERSITY

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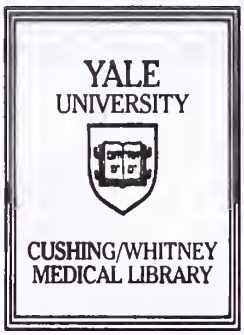
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Urinary Survivin and the Detection of Recurrent Transitional Cell Carcinoma of the Bladder

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by
Justin J. Cohen
2003

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ABSTRACT

Urinary Survivin and the Detection of Recurrent Transitional Cell Carcinoma of the Bladder. Justin J. Cohen, Derek A. Hausladen, Marcia A. Wheeler, Christopher J. Cutie, M. Grey Maher, John W. Colberg, and Robert M. Weiss. Section of Urology, Department of Surgery, Yale University School of Medicine, New Haven, CT.

We investigated whether urinary survivin, an inhibitor of apoptosis protein expressed in many human cancers but few normal adult tissues, could be used as a non-invasive method of detecting recurrence of transitional cell carcinoma (TCC) of the bladder. Urine samples were collected from 121 patients, 98 with treated TCC in remission and 23 with recurrent bladder cancer, and analyzed for survivin content using a novel, antibody based detection system. Mean normalized survivin levels were significantly higher in patients with recurrent TCC than in patients with TCC in remission [0.54 ng/mL(±1.17, n=23) vs. 0.07 ng/mL (±0.15, n=98), $p<0.001$]. Further, patients with invasive recurrent tumors had significantly higher survivin levels than patients with superficial recurrent tumors [1.35 ng/mL (±1.86, n=6) vs. 0.25 ng/mL (±0.67, n=17), $p=0.012$] and patients in remission [1.35 ng/mL (±1.86, n=6) vs. 0.07 ng/mL (±0.15, n=98), $p<0.001$]. The difference between survivin values of superficial recurrent tumors and tumors in remission was not significant [0.25 ng/mL (±0.67, n=17) vs. 0.07 ng/mL (±0.15, n=98), $p=0.36$]. The overall sensitivity of the assay for recurrent bladder cancer in this series is 52.2%, and the specificity is 84.7%, at a survivin value of 0.12 ng/mL. The sensitivity ranges from 41.2% for identifying superficial recurrence to 83.3% for identifying invasive recurrence. Urinary survivin analysis thus appears to be a specific noninvasive method of monitoring for bladder tumor recurrence.

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This thesis is dedicated to the memory of Donald J. Cohen, M.D., who inspired me as a mentor, uncle, role model, and friend.

I thank my parents, whose support and guidance throughout my life have been instrumental in helping me achieve my dreams.

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Introduction

Epidemiology

Cancer remains second only to cardiovascular disease as the leading cause of death in the United States. From 1992 through 1998, cancer death rates declined for both males and females, and incidence rates declined for males but increased for females. During this time period, overall rates, if one takes into account males and females, for both incidence and mortality of cancer declined by an average of 1.1 percent each year. However, despite these improvements, men have a 43.39 percent and women a 38.25 percent chance of developing cancer during their lifetime (1).

It was expected that 56,500 cases of urinary bladder cancer were to be diagnosed in the United States in 2002, with a male to female ratio of 2-2.5 to 1 (41,500 male cases and 15,000 female cases). This makes bladder cancer the fourth most common and tenth most common site of new cancer diagnoses in men and women, respectively, accounting for 7% of the newly diagnosed cancer cases in men and 2% of the newly diagnosed cancer cases in women. It is the second most frequent tumor of the genitourinary system. Bladder cancer was estimated to cause 8,600 male and 4,000 female deaths during that same year, making it the ninth most common cause of cancer death in men (1).

Even though the incidence of bladder cancer and mortality due to this disease remain high, survival rates of those affected by it have increased significantly over the past 25 years. While bladder cancer patients in the United States could expect only a 73% chance of survival at five years between 1974 and 1976, the five-year survival rates

increased to 78% between 1983 and 1985, and 81% between 1992 and 1997. Similar trends were seen both in the white (74% five-year survival between 1974 and 1976; 82% between 1992 through 1997) and African American (48% five-year survival between 1974 and 1976; 65% between 1992 through 1997) populations (1).

The majority of bladder tumors in the United States and Northern Europe are transitional cell carcinomas (TCC), comprising greater than 90 percent of reported cases. Squamous cell carcinoma, adenocarcinoma, and undifferentiated carcinoma make up the remainder of bladder cancers in these regions. Prevalence of TCC peaks in the sixth and seventh decades (2). In contrast, bladder cancer is the most common cancer in areas of the world with endemic schistosomaisis, such as Egypt, and the majority of these cancers are squamous cell carcinomas peaking in the fourth and fifth decades of life (2, 3).

Many environmental factors have been shown to increase the risk of developing bladder cancer. In 1895, Rehn showed an association between dye workers who worked with aniline dyes and bladder cancer, making it the first cancer to be associated with industrialization (4). Since that time, various aromatic amines have been associated with an increased risk of bladder cancer, including those found in hair dyes, cigarette smoke, and the rubber, textile and chemical industries (4, 5).

The most significant environmental risk factor is cigarette smoking, which is estimated to cause up to 65 percent of bladder cancers in males and 25 percent in females (6-8). Smoking increases the risk of developing bladder cancer at least three-fold, with those who smoke for longer periods of time at higher risk than those who smoke less. Aromatic amines, tobacco hydrocarbons and tar are the chemicals in cigarette smoke

implicated in causing the high cancer rates in smokers (5), and patients who are slow acetylators of aromatic amines are at an increased risk of developing bladder cancer (7).

Other factors have been shown to increase the risk of developing bladder cancer. Food products such as coffee, artificial sweeteners and water contaminants are thought by some to be carcinogenic in the bladder. The drugs phenacetin, chlornaphazine and cyclophosphamide have been strongly implicated in oncogenesis. The parasite *Schistosoma haematobium* has been shown in Africa to cause squamous cell bladder cancers. Further, recurrent urinary tract infections and certain genetic polymorphisms are possible causes of bladder cancer (4-6, 9).

Pathology of TCC

The luminal lining of the bladder is composed of a transitional cellular epithelium which allows the bladder to distend and accommodate large volumes of urine. Deep to this urothelium lies a basal lamina, separating it from the lamina propria. The lamina propria contains vessels, lymphatics and nerve fibers. After the lamina propria comes a muscularis mucosa, which contains a layer of medium sized vessels. The submucosa borders this layer, separating it from the detrusor muscle, a layer consisting of larger interlacing bundles of smooth muscle (10).

Transitional cell carcinomas are staged according to the TNM system (Table 1). This system is of limited value in most bladder cancers, because the typical treatment for bladder tumors is transurethral resection, and one cannot determine lymph node involvement nor distant metastases from such specimens. Further, resection specimens

are limited in the diagnosis of pathologic tumor stage. It is possible to determine whether invasion into muscle has occurred, but determination of whether the tumor has invaded perivesical fat or adjacent organs is not possible.

Grading of TCC is more subjective than staging, with less reproducible results (11). Many grading systems are in use, and two new classification systems, the WHO/ISUP 1998 and WHO 1999 systems, have recently been published to avoid overdiagnosis of cancer and to create better criteria for the grades (12). These systems grade neoplasms based on the degree of order in the architectural pattern and distinction of variation of the cellular features building that architecture. According to the WHO 1999 system, tumors with predominant order in the architectural pattern are considered papillary urothelial neoplasms of low malignant potential or grade I tumors, depending on the amount of variation in the architecture. Tumors which are predominantly disordered are considered high grade carcinomas. These are categorized as grade II urothelial carcinomas if focal order is seen within the tissue, and grade III carcinomas if no focal order is seen within the tissue (12).

Natural History of TCC

Transitional cell carcinoma (TCC) of the urinary bladder may behave in four ways if simple resection and fulguration are the sole means of treatment. There may be no further recurrence of the tumor, the tumor may recur once or multiple times but without change in pathologic stage, the tumor may progress in stage locally within the

bladder, or it may spread to distant sites within the body (11, 13). Tumors presenting in different stages and with different grades have varied rates of recurrence.

Most bladder cancers are superficial; that is, tumors not invading muscularis propria, stages Tis, Ta, T1a, and T1b. Superficial, well-differentiated papillary TCC account for 55-85 percent of tumor presentations (13). Most superficial papillary TCC present as solitary tumors, but 30 percent of patients may have multiple tumors (14).

Heney *et* al (15) reported on 249 patients in the National Bladder Project Collaborative Group A who received only transurethral resection and fulguration of their tumors prior to the first recurrence. Four percent of patients who initially presented with Ta disease progressed to muscle invasive or metastatic disease, while 30 percent of patients with T1 disease progressed. Similarly, 2 percent of grade 1 tumors progressed, while 11 percent of grade 2 and 45 percent of grade 3 tumors progressed. Of those patients with stage T1, grade 3 disease, there was a nearly 50 percent risk of progression in this study. Progression occurred within two years in most cases.

In 1986, Fitzpatrick *et al* (16) reported on a series of patients with Ta, grades 1 and 2 bladder tumors. They found that after 5 or more years of follow-up, 54 percent had developed recurrent tumor. Only 6 percent had a worsened tumor stage. Forty percent of those with recurrent tumor had only one recurrence whereas 60 percent of those with recurrence had more than one recurrence. Patients in the study who had no evidence of tumor recurrence 3 months after initial resection and fulguration had an 80 percent chance of remaining tumor-free. Those that did have recurrent tumor at 3 months follow-up had a high probability of further recurrences. Furthermore, patients with multiple

tumors at initial presentation and those with tumors of 10 grams or greater at initial presentation tended to have a worse prognosis.

In a study of 178 patients with stage Ta, grade 1 lesions in the National Bladder Project Collaborative Group A who underwent resection and fulguration of bladder tumors as the sole means of treatment, Prout Jr. *et al.* (17) reported recurrences in 61 percent of patients followed for a median of 58 months. When their tumors recurred, 27 percent of patients exhibited grade progression while only 4.5 percent showed stage progression.

Recurrence rates and stage progression of stage Ta, grades 1 and 2 tumors are important because Ta disease is a relatively benign disease, and patient survival is similar to that of age- and sex-matched controls (11). Although the grade and stage of TCC do not allow for absolute characterization of whether a tumor will recur and, if it does recur, progress to a more invasive tumor, the available evidence overwhelmingly suggests that higher grade tumors and increased stage superficial tumors are more likely to be recurrent and progressive than lower grade and stage tumors.

Although carcinoma in situ is classified as a superficial TCC, its natural history is quite different than Ta and T1 superficial TCC. Fifty percent of patients with primary carcinoma in situ will die of metastatic bladder cancer within two years unless aggressive treatment with intravesical therapy, and cystectomy if that fails, is pursued. Because of its dismal prognosis and high rates of progression, CIS appears to be a precursor of invasive bladder TCC.

Invasive TCC, stages T2-T4, accounts for 15-20 percent of transitional cell carcinomas, and the muscle invasion is present upon initial tumor presentation in 60-80

percent of these patients (14). Invasive tumors are typically not candidates for transurethral resection, and thus cystectomy is the treatment of choice for locally advanced disease (18). Fifty percent of those with muscle invasive disease will develop metastases (19), and the 5-year survival rate of T2-T3 disease is 20-40 percent (14, 20).

The behavior of transitional cell carcinoma of the bladder seems such that superficial tumors should be resected and treated, recurrences, especially those with advanced stage, should be identified promptly, and invasive tumors should be dealt with promptly and aggressively.

Diagnosis

The primary symptom of eighty percent of patients with TCC of the bladder is intermittent hematuria (6, 21, 22). Other common presenting symptoms include irritative voiding symptoms such as dysuria, frequency, nocturia and urgency, flank pain, abdominal pain and weight loss (22). In the absence of evidence of a medical cause of the hematuria such as proteinuria, red blood cell casts or elevated creatinine, patients with hematuria are worked-up for urologic causes such as bladder cancer.

Grossfeld *et al* (23) reported the American Urological Association Best Practice Policy for the evaluation of asymptomatic microscopic hematuria and elucidated guidelines for investigating whether those with hematuria without an obvious medical cause may be secondary to bladder cancer. Following an in-depth history and physical exam, urinalysis and culture is recommended to rule out obvious medical and benign causes of hematuria. Next, a complete evaluation includes radiologic imaging of the

upper genitourinary tract followed by cystoscopic examination of the bladder in appropriate patients. Intravenous urography (IVU) has traditionally been considered the best initial radiological study, but computed tomography, magnetic resonance imaging and ultrasound are also used by some practitioners. Flexible cystoscopy should be performed on all adults older than 40 years and those younger than 40 years with risk factors for bladder cancer. In addition to cystoscopy in some patients, and in all patients who do not receive cystoscopy, urinary cytology should be performed as a highly specific screening tool for bladder cancer. If bladder cancer is suspected from these tests, confirmation should be made with appropriate radiological measures and/or rigid cystoscopy, and treatment decisions should be made following accurate staging of the tumor.

New Methods of TCC Detection

While the combination of cystoscopy and cytology remains the gold standard for evaluation of TCC, intense research is underway to develop new tests that may prove more useful. The ideal test would be a simple, inexpensive, noninvasive point of use test with high sensitivity and specificity for new and recurrent bladder cancer. Although early detection of all bladder tumors would theoretically be the goal of such a test, the low prevalence of bladder cancer in the general population and the low proportion of high grade and invasive tumors makes general population screening impractical due to cost and the number of false-positive results that would ensue (24). Thus, a more practical

use of such tests would be to investigate hematuria, screen high risk individuals, and screen patients for recurrence of bladder tumors.

Monitoring tumor recurrence would be an important function of any useful bladder tumor marker. Earlier diagnosis of a cancer is thought to improve outcome inasmuch as treating a smaller cancer should lead to improved cure rates (25). Since greater than 50 percent of those presenting initially with superficial tumors will develop recurrent tumors (16), and recurrence is often accompanied by progression to a more invasive tumor, patients are currently followed by cystoscopy every 3 to 6 months for at least one year, and yearly thereafter, in an attempt to detect recurrence while it is still treatable. This method of surveillance is expensive, uncomfortable for the patient, has a risk of morbidity, and may not detect flat lesions. A urinary tumor marker test, if proven to be as sensitive and specific as cystoscopy and cytology, could improve patients' experience and outcome by offering an affordable, non-invasive (and therefore morbidity-free), accurate test that would predict or at least identify tumor recurrence.

Many such tests are currently available or under development (table 2), but none of them have yet proven to be a replacement for cystoscopy or cytology. Like cytology, most of these assays are highly specific to TCC of the bladder. The results of the many studies on these markers show that nearly all of the currently available assays have a comparable or higher sensitivity than cytology, but a lower specificity (26), and thus are not suitable replacements at this time. The methods that are not currently commercially available, including telomerase, BLCA-4, hyaluronic acid/hyaluronidase and survivin are promising alternatives to cytology and cystoscopy, and are currently under investigation.

Survivin

Apoptosis, or programmed cell death, is governed by networks of pathways which induce or prevent cell death in order to achieve tissue homeostasis. This cell death is mediated by intracellular proteases called caspases, and results in membrane-encased fragments of cells which are cleared by phagocytosis. Dysregulation of processes controlling apoptosis is thought to contribute to neoplastic cell expansion and carcinogenesis (27).

One family of apoptosis regulators, the Bcl-2 family, is comprised of apoptosis-suppressing and apoptosis inducing members. Alterations in their expression due to structural changes in the genes or changes in transcriptional and post-transcriptional regulatory networks may cause imbalances in the ratio of anti-apoptotic to pro-apoptotic proteins, leading to cells that are resistant to death stimuli (27).

Another family, the inhibitors of apoptosis protein (IAP) family, was first described in bacloviruses in 1993 (28). This widely expressed family is thought to suppress apoptosis through direct caspases and pro-caspase inhibition, primarily caspases 3 and 7, and interaction with NF-κB (29). IAPs are thought to contribute to oncogenesis and tumor progression, and the strongest evidence to support this notion is found in data that have accumulated about the IAP survivin (29).

Survivin, a member of the IAP family first described in 1997 by Ambrosini *et al* (30), is a unique member of the IAP family which contains a single baculovirus IAP repeat and lacks a carboxyl-terminal RING finger, which is found in certain IAPs (31, 32). In their initial paper, Ambrosini *et al* noted expression of survivin in fetal tissues,

lung, colon, pancreas, prostate and breast cancers, and high-grade non-Hodgkin's lymphomas, but not terminally differentiated adult tissues nor low-grade lymphomas (30). Since that time, it has also been found to be over-expressed in neuroblastoma (33), leukemia (34), pheochromocytoma (35), glioma (36), melanoma (37), and gastric (38), esophageal (39), hepatic (40), uterine (41), ovarian (42), and bladder (43) cancers. Furthermore, studies have found that survivin is expressed in select non-cancerous tissues, including prostatic neuroendocrine cells (44), proliferating endometrium (45), and quiescent $CD34^+$ bone-marrow-derived stem cells (46).

Survivin and Bladder Cancer

Because survivin is overexpressed in many tumors but not in most normal tissues, Swana *et al* (43) investigated whether it was expressed in bladder tumor tissue. To that end, they immunohistochemically studied the primary tumors of 36 patients with localized bladder cancer for expression of survivin, bcl-2, and p53. The subjects included 25 men and 11 women with a mean age of 66 years. Survivin was detected in 28 of the 36 tumors studied, including 65 percent of grade 1 tumors, 90 percent of grade 2 tumors, and 100 percent of grade 3 tumors. Survivin was not detected in normal bladder urothelium. Furthermore, patients with grade 1 tumors staining survivin-positive recurred more quickly (12±6 months) than patients whose grade 1 tumors did not stain for survivin (36±16). Staining for bcl-2 or p53 was not correlated with disease recurrence.

Since survivin was detected in bladder tumor tissue and not normal bladder urothelium, Smith *et al* (47) performed preliminary studies to determine whether survivin could be detected in the urine of patients with bladder cancer. Urine specimens were collected from healthy volunteers (n=17), patients with nonneoplastic urinary tract disease (n=30), genitourinary cancer other than TCC(n=30), new-onset or recurrent bladder cancer (n=46), and successfully treated bladder cancer, as determined by negative post-treatment cystoscopy (n=35). Samples were analyzed using an antibody-based, dot-blot assay, Western Blot and reverse transcriptase polymerase chain reaction. Sensitivity for new-onset and recurrent bladder cancer was 100 percent, and specificity for other neoplastic and nonneoplastic genitourinary tract diseases was 95 percent. This high sensitivity and specificity test showed promise in this study as a potential tumor assay for both new and recurrent bladder cancer.

Urinary testing for survivin appears to be a promising method of surveillance for bladder cancer. Because recurrent cancer, especially recurrent TCC which has increased in grade or become more invasive, is a major problem facing urologists and bladder cancer patients, and because the current methods of surveillance have significant drawbacks and limitations, we hypothesize that urinary survivin measured by the Bio-dot antibody based assay is a highly sensitive and specific marker for high grade, invasive TCC of the bladder.

Statement of Purpose and Hypothesis

Urinary testing for survivin appears to be a promising method of surveillance for bladder cancer. Because recurrent cancer, especially recurrent TCC which has increased in grade or become more invasive, is a major problem facing urologists and bladder cancer patients, and because the current methods of surveillance have significant drawbacks and limitations, we hypothesize that urinary survivin measured by the Bio-dot antibody based assay is a highly sensitive and specific marker for high grade, invasive TCC of the bladder. We hope that this technique will prove to be an effective method for predicting and diagnosing recurrent TCC.

Methods

Urine Specimens

121 clean-catch or straight catheter urine samples were collected at the urology clinics at Yale-New Haven Hospital and the Veterans Affairs, New England Health Care Systems, West Haven, Connecticut, Division. These samples were taken from patients with a pathologically documented history of bladder cancer which was treated at least two months prior to sampling. The nursing staff and house officers at these institutions obtained the samples. Urine samples were aliquoted and stored in aliquots at -80°C until analysis.

Urine detection of survivin

The protocol is as previously published (47) except for the use of longer incubation times with the primary antibody, a secondary antibody to a fragment of IgG which was more sensitive and specific than the antibody to holo-IgG, longer times for detection of the chemilluminescent product, and the use of frozen whole urine samples. Frozen whole urine samples frequently contain more survivin than urine samples that are centrifuged and the supernatants subsequently frozen. On the day of survivin analysis, urine samples were thawed and centrifuged at 20,000 x g for 20 minutes. Meanwhile, the Bio-Dot Microfiltration Apparatus was assembled with a 0.2 μ nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) that had been moistened in 20 mM Tris-buffered saline (pH 7.5). Then, the urine supernatants (500 μl), along with increasing

concentrations (0.1-100 ng/ml) of E.coli-expressed recombinant survivin as a standard (48), survivin-negative protein controls and survivin-positive and negative control urine samples were filtered onto the membrane.

After filtration, the membrane was incubated (2 hours, 4°C) with 5% nonfat dry milk and 0.01% sodium azide in PBS, pH 7.4. After washing in PBS-Tween 20 (0.5%), the membrane was incubated overnight at 4°C, with 2 μg/ml of a rabbit polyclonal antibody to survivin (37), washed with PBS-Tween (0.05%), and incubated with a 1:24,000 dilution of a horseradish peroxidase-conjugated donkey anti-rabbit F(ab')2 fragment of IgG (Jackson ImmunoResearch, West Grove PA) (1 hour, 22C). After two washes each in PBS, PBS-Tween (0.05%) and PBS again, binding of the primary antibody was detected by enhanced chemilluminescence (Amersham Biotech, Piscataway NJ). Blots were scanned and concentrations of survivin were determined using the Kodak Digital Science 1D Image Analysis System (Eastman Kodak Co., Rochester, NY). This was followed by determination of a survivin value for each sample based on the antibody reactivity relative to six survivin standards (0.1-100 mg/ml).

Urine sample database

The urine samples were collected between 2000-2001, and were analyzed by the laboratory in a blind manner. Because of this, the diagnosis of each patient at the time urine samples were obtained was unknown. Unfortunately, since bladder cancer is a complex disease with multiple methods of diagnosis and a tendency to recur months to years post-treatment, the classification of patients' diagnosis for each urine sample could

not be completely described with a simple evaluation of pathology reports. We therefore decided to create a database of the entire urological medical record for each patient enrolled in the study.

We specifically constructed a database to be used to categorize all patients with hematuria, new-onset bladder cancer, bladder cancer in remission, and recurrent TCC using FileMaker Pro 5.0 (FileMaker, Inc., Santa Clara, CA). We designed this database to contain all of the information required to accurately assess the bladder cancer status of each patient at any given time, including cystoscopy, cytology, and therapeutic information. In addition, we included demographic data, information about the patient's prostate and renal status, results of all imaging studies performed on the patient, and any other information which might contribute to the patient's medical status. This information should also make the database quite useful in other studies involving urologic patients at our institutions.

Since the database was to contain such a large volume of data (much of the above mentioned data were repeated multiple times for each patient, and all such studies in each patient's medical record were included in the database), we used forms to create the appearance of multiple pages for each record, all linked by a background of the patient's demographic data (Figures 1 and 2). Tabs were created to easily switch between forms, and navigation buttons were included at the top of the database to allow for quick navigation between records.

Once the database was created, we surveyed the medical records of all patients from whom urine samples were obtained and entered all of the appropriate data into the database. Efforts were made to ensure that the medical record was complete for each

patient, and only diagnoses of recurrent cancer which were verified by pathological data from cystoscopy biopsy specimens were included in the study. Samples from patients in remission without recurrence within one year after the sample was given were included in the remission group, and patients with cystoscopic biopsies with pathology positive for TCC at the time of urine sample were included in the recurrence group. All patients included in the study had at least one year of follow-up.

Data analysis and statistics. Assignment of recurrence and remission status is determined by post treatment cystoscopy (± biopsy). Differences between groups were determined using Statview (SAS Institute, Cary, NC). Study results were expressed as the mean ± standard error, and as the median. Outcome of treatment was analyzed using non-parametric analysis including the signed t test and Mann-Whitney U tests. The detection limit of the assay is 0.03 ng/ml survivin with no nonspecific background, and all values below this level were treated as 0.

Exclusionary criteria

All patients from whom urine samples had been reserved were initially eligible for inclusion in this study. Those patients with recurrent bladder cancer and/or bladder cancer in remission were selected from the database for this analysis. Samples from patients undergoing intravesical chemotherapy or immunotherapy were excluded due to possible confounding effects of this therapy on survivin levels (Hausladen et al, *in press*). Patients who had visual recurrence of tumor without biopsy-proven pathological data to

document TCC were excluded because some patients with visual tumor recurrence had negative pathology specimens. All patients with biopsies negative for TCC were included in the remission group. Patients who were in remission at the time that the sample was gathered but developed recurrent TCC during the follow-up period were excluded in the study.

The author performed two of the Bio-Dot assays. Marcia A. Wheeler did the remainder of the assays and all of the digital survivin quantification. J.J.C., C.J.C., and D.A.H. were primarily involved in the pre-design planning of the database. J.J.C. implemented the database, and J.J.C., C.J.C., and D.A.H. acquired data. J.J.C. performed the statistical analysis.

Results

Determination of urine survivin with the Bio-Dot apparatus was performed on 121 samples. Eighty-nine of these samples were taken from men in remission from TCC and nine from women in remission All of the patients in remission were free of tumor for at least one year after the sample was obtained. Twenty samples represented men with recurrent TCC, and three samples were taken from women with recurrent cancer. The mean age (±SD) of patients in the remission group was 68.2 (±12.4) years and the mean age of those patients with tumor was 72.0 (±10.9) years (Table 4). Of those with recurrent TCC, 16 males and one female had superficial recurrent disease while four males and two females have invasive recurrent disease. Eleven men and one woman had low-grade recurrence, while nine men and two women had high-grade recurrence (Table 5).

Survivin was detected in 34 of the 98 (34.7%) samples from patients with TCC in remission and 12 of the 23 (52.2%) samples from patients with recurrent disease (Table 3). It was detected in 7 of the 17 (41.2%) cases of superficial recurrence and 5 of the six (83.3%) cases of invasive recurrence, and 6 of 12 (50%) cases of low-grade recurrence and 6 of 11 (54.5%) cases of high-grade recurrence (Table 3).

When normalized for a weighted mean (SD) survivin level, patients with recurrent TCC had significantly higher survivin levels than patients in remission [0.54 ng/mL(±1.17) vs. 0.07 ng/mL (±0.15), $p<0.001$] (Table 4).

The correlation between survivin score and histopathologic grading and staging is shown in Table 4 and graphically depicted in Figures 3 and 4. Survivin values for high

grade recurrent tumors were significantly higher than survivin values for patients in remission [0.75 ng/mL (±1.49) vs 0.07 ng/mL (±0.15), p=0.029], but there were no statistically significant differences between the survivin levels of low grade recurrent tumors vs. remission and the survivin levels of high grade recurrent tumors vs. low grade recurrences. Survivin values for invasive recurrent tumors were significantly higher than survivin values of patients in remission [1.35 ng/mL (±1.86) vs. 0.07 ng/mL (±0.15), p<0.001] and survivin values of patients with superficial recurrence [1.35 ng/mL (±1.86) vs. 0.25 ng/mL (±0.67), p=0.012].

Using a threshold value of 0.12 ng/mL survivin, this test is 84.7% specific for TCC in remission. The overall sensitivity of this assay for recurrent TCC of the bladder is 52.2% (Table 6). The sensitivities for low grade recurrence and high grade recurrence are 50.0% and 54.5%, respectively. The sensitivities for superficial recurrence and invasive recurrence are 41.2% and 83.3%.

Discussion

In this study, we investigated whether an antibody-based urinary assay for the inhibitor of apoptosis survivin could be used to differentiate whether patients with previously treated bladder cancer remained in remission or had recurrent bladder tumors. We found that patients with recurrent TCC of the bladder had significantly higher urinary levels of survivin than patients with TCC in remission. This effect is most pronounced when comparing the levels in patients with invasive recurrence and high grade recurrence to those patients in remission.

Survivin was detected in 34 of the 98 (34.7%) samples from patients with TCC in remission and in 12 of the 23 (52.2%) samples from patients with recurrent disease (Table 3). It was detected in 7 of the 17 (41.2%) cases of superficial recurrence and 5 of the six (83.3%) cases of invasive recurrence, and 6 of 12 (50%) cases of low-grade recurrence and 6 of 11 (54.5%) cases of high-grade recurrence.

The overall sensitivity of the assay for recurrent bladder cancer in this series is 52.2% at a cutoff survivin value of 0.12 ng/mL, and the specificity is 84.7% at this threshold. The sensitivity ranges from 41.2% for identifying superficial recurrence to 83.3% for identifying invasive recurrence.

Previous pathologic studies of survivin (43) had shown that survivin was detected in 28 of the 36 tumors studied, including 65 percent of grade 1 tumors, 90 percent of grade 2 tumors, and 100 percent of grade 3 tumors, and was not detected in normal bladder epithelium. Our data agree with those of Swana *et al*, in that urinary survivin was detectable in the urine of greater than 50 percent of our cohort with recurrent disease,

and a higher proportion of urines were positive for survivin in more aggressive (higher grade and invasive) tumors. Although Swana *et al* did not detect survivin in normal bladder urothelium, 34.7% of patients in remission in our series had detectable urinary levels of survivin. Although these patients were free of recurrence for one year after we collected their samples, it is possible that these patients will be more likely to be in the cohort of greater than 60% of bladder cancer patients who develop recurrent tumor. Also, the normal epithelium that Swana *et al* studied had not had prior episodes of TCC.

Smith *et al* (47) found this Bio-dot assay to be 100 percent sensitive for new-onset and recurrent bladder cancer, and 95% specific for other neoplastic and nonneoplastic genitourinary disease. Our specificity of 84.7% is nearly the level that Smith *et al* found, which compares favorably to existing tests and is more impressive since our controls were all patients who had previously been treated for TCC, while other methods of detecting bladder cancer have traditionally been tested comparing samples from cancer patients with samples from patients without a history of TCC. The sensitivities obtained in our study were considerably lower than reported in that previous study, with the exception of invasive recurrent TCC. This was not entirely unexpected, however. Histopathologic analysis of bladder epithelium by Swana *et al* only found survivin in 65% of epithelium with grade 1 lesions. Further, the collection of urine samples in this study was not ideally standardized. We did not determine the amount of time that urine basted in each patient's bladder before collection. It seems reasonable to assume that urine with more contact time with urothelium containing a bladder tumor would have a higher likelihood of staining for survivin in the dot-blot assay. To decrease this

confounding factor, urine samples in future studies would ideally be collected by patients as a first morning void on the date of their clinic appointment.

The retrospective design of our database and method of sample collection unfortunately did not allow us to determine whether urinary survivin could predict recurrence of TCC. Although we analyzed the urine of patients in remission, only five of those patients progressed to having recurrent tumors after the date we collected the urine. Additionally, we did not aliquot urine samples for those patients when they did recur, making comparison of survivin levels impossible. A design change in future studies would correct this and allow us to determine the predictive accuracy of survivin for recurrence. If we analyzed urine samples at every visit of all bladder tumor patients seen in the clinics, we would be able to better determine whether urinary survivin could predict as well as identify TCC.

Although cystoscopy in conjunction with cytology is the gold standard for bladder tumor follow-up, less than half of the survivin samples which we included in our study had cytology results in addition to cystoscopy results. Because of this, we were unable to compare our method for identifying bladder tumor recurrence with cytology. Future studies should assure that urine samples are sent for cytologic analysis in addition to survivin. Also, since we did not have cytology data for all of the patients, and cystoscopic biopsies were only commonly obtained on patients with visual lesions, it is possible that some patients whom we included in the remission group actually had recurrent TCC at the time the urine sample was taken. This would cause the calculated sensitivity and specificity values to be lower than their actual values.

Finally, the large number of subgroups in our study and relative frequencies of different grades and stages of TCC caused the number of patients in some of our groups to be low; for instance, there were only 6 patients in this study with recurrent, invasive tumors. A larger study in the future may allow us to break these groups down even further and detect finer variations in the level of survivin.

The search for a highly sensitive and specific, easily accessible marker for bladder cancer remains an elusive tool in the treatment of TCC. Many such tests are currently available or under development, but none of them have yet proven to be a replacement for cystoscopy or cytology. The inhibitor of apoptosis survivin is overexpressed in many human cancers including TCC of the bladder, and may prove to be a useful molecular marker in diagnosing and predicting recurrence of TCC. We showed that urinary survivin values are significantly higher for patients with recurrent TCC than patients with TCC in remission. This test is highly sensitive for cancer in remission, and is highly specific for invasive recurrent tumors. Future studies may be able to determine whether urinary survivin could be used as a tool to predict TCC recurrence in place of or in conjunction with cystoscopy and cytology.

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Table 1. TNM Staging of Bladder Carcinoma

Primary Tumor (T)		
Tx		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Ta		Non-invasive papillary carcinoma
Tis		Carcinoma in situ
T1		Tumor invades sub-epithelial connective tissue
	T1a	Basement membrane penetration
	T1b	Lamina propria invasion
T2		Tumor invades the detrusor muscle
	T2a	Superficial (inner half) muscle invasion
	T2b	Deep (outer half) muscle invasion
T3		Tumor invades the perivesical tissue
	T3a	Microscopic invasion
	T3b	Macroscopic invasion
T4		Tumor invades any of the following:
	T4a	Prostate, uterus, vagina
	T4b	Pelvic wall, abdominal wall

Regional Lymph Nodes (N)	
Nx	Regional lymph nodes not assessed
N0	No regional lymph node metastases
N1	Single nodal metastases (<2 cm)
N2	Single or multiple nodal metastases (none >2-5 cm)
N3	Nodal metastases (>5cm)

Distant Metastases (M)	
Mx	Distant metastases not assessed
M0	No distant metastases
M1	Distant metastases

modified from Neal and Griffiths, in Management of Urologic Malignancies (2002)

Table 2. Current Bladder Tumor Marker Tests

Test	Marker Detected	Specimen	Assay Type	% Sensitivity	% Specificity	Testing Environment
			Commercially produced			
Cytology	Cell morphology	Urine, Barbotage	Pathologic analysis	35-61	90-100	Specialized laboratory
Hemastix hematuria	Hemoglobin	Urine	Dipstick	67-90	Low	Point of care
BTA Stat, BTA TRAK	Human complement factor H related protein, complement factor H	Urine	Immunoassay	57-83	46-90	Specialized laboratory, Point of care
NMP22	Nuclear mitotic apparatus	Urine	ELISA	47-100	60-82	Specialized laboratory
Accu-Dx	Fibrin-fibrinogen degradation product, fibrin, fibrinogen	Urine	Immunoassay	33-83	75-86	Point of care
Immunocyt	Mucins, high molecular weight carcinoembryonic antigen	Urine, exfoliated cells	Immunofluorescence	86	79	Specialized laboratory

Not yet commercial						
Telomerase	Telomerase human telomerase messenger RNA	Urine, exfoliated cells	Telomeric repeat amplification protocal assay, human telomerase RT-PCR	62-86	60-96	Specialized laboratory
HA-HAase	Hylauronic acid, hyaluronidase	Urine	ELISA-like	92-100	84-93	Specialized laboratory
Quanticyt	Nuclear shape, DNA content	Barbotage, exfoliated cells	Image analysis	45-70	70-93	Specialized laboratory
Fluorescence in situ hybridization	chrosomal analysis	Urine, barbotage	Immunofluorescence	73	100	Specialized laboratory
BCLA-4	Nuclear matrix protein	Urine	Immunoassay	96.4	100	Specialized laboratory
Survivin	Inhibitor of Apoptosis Protein	Urine	Dot-blot immunoassay			Specialized laboratory

Modified from Lokeshwar (2001) and Konety (2001)

Table 3. Numbers of samples which had detectable survivin for each pathologic group.

Group	Survivin Positive (n)	Survivin Negative (n)	% Survivin Positive
Remission (n=98)	34	64	34.7
All Recurrent Tumors (n=23)	12	11	52.2
Low Grade Recurrence (n=12)	6	6	50.0
High Grade Recurrence (n=11)	6	5	54.5
Superficial Recurrence (n=17)	7	10	41.2
Invasive Recurrence (n=6)	5	1	83.3

Table 4. Comparison of Patients in Remission and All Patients with Recurrent Tumor

	Remission		Recurrent Tumor	
			All Tumors	
	N (%)	Mean(SD)	N(%)	Mean(SD)
Gender				
Male	89 (81.7)		20(18.3)	
Female	9 (75)		3(25)	
Age				
(Years)		68.2(12.4)		72.0(10.9)
Cytology				
normal	27(90)		3(10)	
atypical	20(100)		0(0)	
positive	6(66.7)		3(33.3)	
Survivin				
mean (µg/mL)		0.07(0.15)		0.54(1.17)*

*Significantly greater than survivin level for remission (p=0.00)

Table 5. Comparison of Patients in Remission and Patients with Recurrent Tumor, By Group

	Remission		Recurrent Tumor							
			Stage				Grade			
			Superficial		Invasive		Low		High	
	N (%)	Mean(SD)	N(%)	Mean(SD)	N (%)	Mean(SD)	N(%)	Mean(SD)	N(%)	Mean(SD)
Gender										
Male	89 (81.7)		16(14.7)		4(3.7)		11(10.1)		9(8.3)	
Female	9 (75)		1(8.3)		2(16.7)		1(8.3)		2(16.7)	
Survivin										
mean (μg/mL)		0.07(0.15)		0.25(0.67)		1.35(1.86)*		0.34 (0.79)		0.75(1.49)**

*Significantly greater than survivin level for both remission and superficial recurrence.

**Significantly greater than survivin level for remission

Table 6. Sensitivity and Specificity of Urinary Survivin for Recurrent Tumors

	Survivin Cutoff	%Sensitivity	%Specificity
Recurrent Tumor	0.119	52.2	84.7
Low Grade Recurrence	0.119	50	84.7
High Grade Recurrence	0.119	54.5	84.7
Superficial Recurrence	0.119	41.2	84.7
Invasive Recurrence	0.119	83.3	84.7

Figure 1.

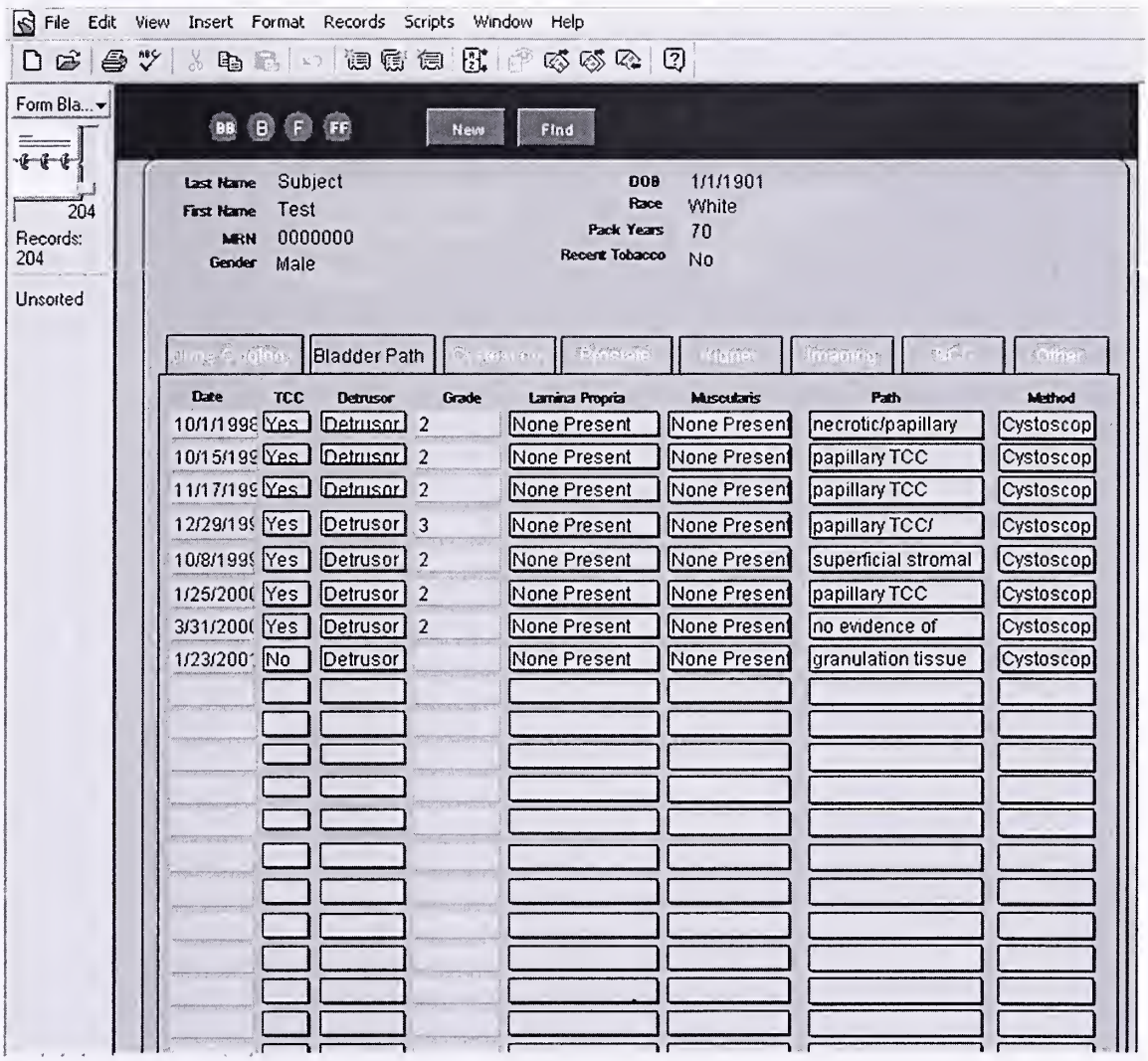

Figure 1. A Representative Entry for Bladder Biopsy Pathology Findings. Demographic information about a test subject appears at the top of all forms within each subject's entry. Tabs, labeled "Urine Cytology," "Bladder Path," "Cystoscopy," "Prostate," "Kidney," "Imaging," "BCG," and "Other" direct the user to the form containing information relevant to each title. This form, which has data regarding a patient's bladder pathology specimens, tells the researcher whether each dated sample contained TCC, the depth of invasion of that cancer, its grade, how the sample was obtained, and any other relevant information contained on the pathology report.

Figure 2.

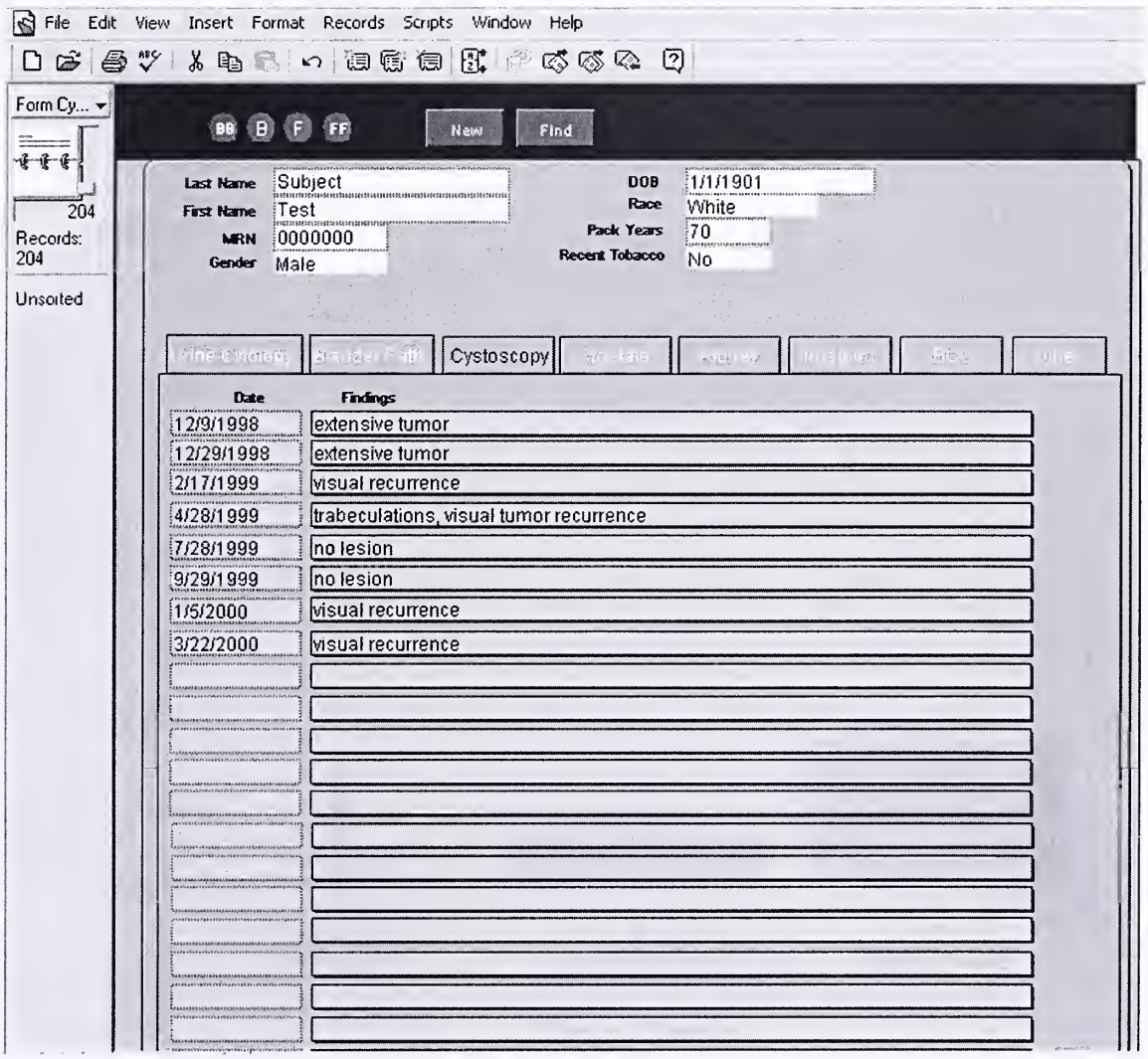

Figure 2. A Representative Entry for Visual Cystoscopic Findings. As in the previous figure, demographic information is at the top of the screen, and tabs allow movement between forms within subject's entry. This form contains dated data for all of the visual cystoscopic findings in this patient's medical record.

Figure 3.

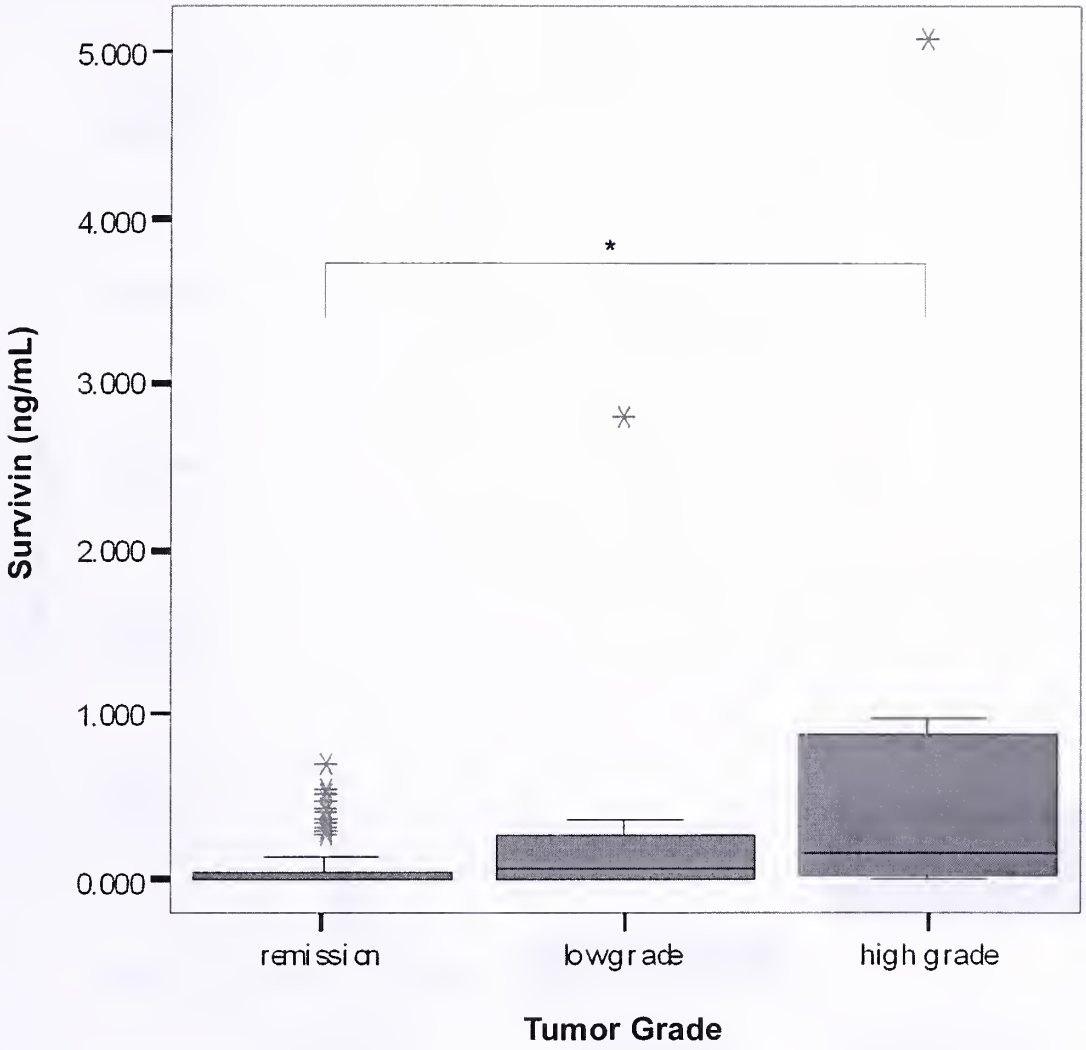

Figure 3. A box and whiskers plot depicting the survivin levels of patients in remission, patients with low grade recurrence and patients with high grade recurrence. Survivin values for high grade recurrent tumors were significantly higher than survivin values for patients in remission *[0.75 ng/mL (±1.49) vs 0.07 ng/mL (±0.15), p=0.029], but there were no statistically significant differences between the survivin levels of low grade recurrent tumors vs. remission and the survivin levels of high grade recurrent tumors vs. low grade recurrences.

Figure 4.

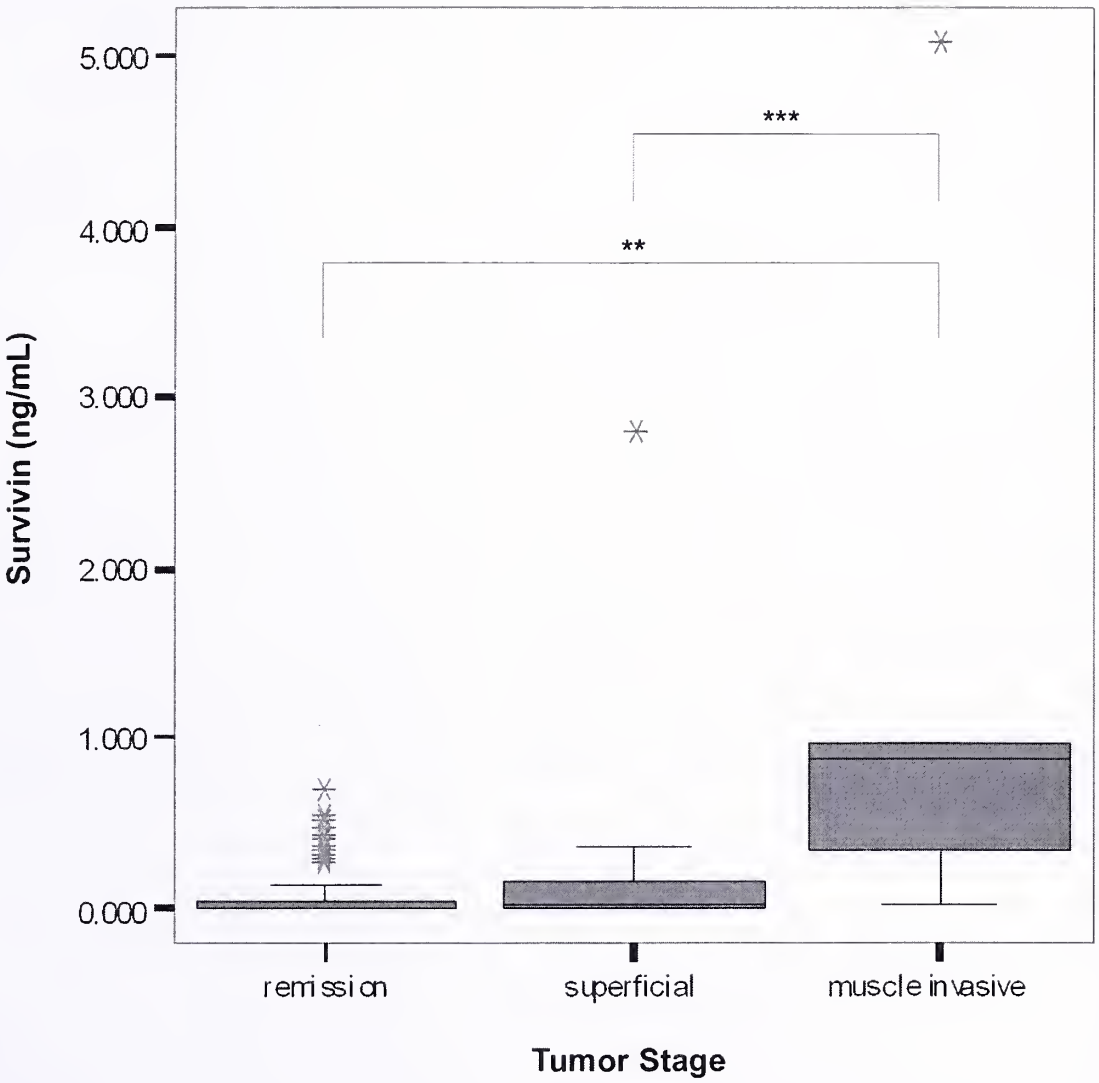

Figure 4. A box and whiskers plot depicting the survivin levels of patients in remission, patients with superficial recurrence and patients with muscle invasive recurrence. Survivin values for invasive recurrent tumors were significantly higher than survivin values of patients in remission **[1.35 ng/mL (±1.86) vs. 0.07 ng/mL (±0.15), p<0.001] and survivin values of patients with superficial recurrence *[1.35 ng/mL (±1.86) vs. 0.25 ng/mL (±0.67), p=0.012]. The difference in survivin values between patients with superficial recurrence and patients in remission was not statistically significant.**

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